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PRELIMINARY INVESTIGATIONS ON THE SINGLE AND COMBINED CYTOTOXIC EFFECT OF T-2 AND HT-2 TOXIN MEASURED BY METHYL THIAZOL TETRAZOLIUM (MTT) CYTOTOXICITY TEST USING PIG LYMPHOCYTES

Veronika RAJLI [1], Zsófia BODNÁR [1], Katalin HORVATOVICH [1], László KAMETLER [1], Michael F. DUTTON [2], Melinda KOVÁCS [1, 3]

ABSTRACT

Fusariotoxins are frequently existing contaminants in cereal and other plant products. High relationship was found between the levels of the trichothecene T-2 and HT-2 toxin detected in contaminated commodities. Many *in vitro* studies investigated the cytotoxicity of T-2 and HT-2, but the interaction between them has not been thoroughly studied yet. The aim of the study was to examine the dose dependent cytotoxic effect of T-2 and HT-2 toxins, single and in combination, using pig lymphocytes in the methyl thiazol tetrazolium (MTT) assay. The mycotoxins were added at various concentrations, i.e. 0.5, 0.1, 0.05, 0.01 and 0.001 μM of T-2; 1.0, 0.5, 0.2., 0.1 and 0.05 μM of HT-2; and both mycotoxins combined. Two exposure times (6 and 24 h) were tested. Both T-2 and HT-2 toxins exerted a dose dependent effect. After 6 h incubation, the increase in concentration of T-2 from 0.001 to 0.5 μM and HT-2 for 0.05 to 1.0 μM resulted in lower cell viability by 22 and 17%, respectively. After 24 h cell viability was significantly lower compared to values obtained at 6 h, except 0.5 μM T-2 and 0.05, 1.0 μM HT-2, respectively. Measured cell viability for combinations of T-2 and HT-2 was higher compared to the calculated expected values. The two toxins caused lower cell survival when applied together than in single administration after 6 h incubation. After 24 h incubation this tendency was not consistent.

Key words: mycotoxins / cytotoxicity / pig lymphocytes / methyl thiazol tetrazolium (MTT) assay

1 INTRODUCTION

In vitro Methyl Thiazol Tetrazolium (MTT) cell culture assay is one of the most frequently used tests for preliminary screening of cytotoxicity of *Fusarium* toxins. It is a rapid, versatile, quantitative, and highly reproducible colorimetric assay for mammalian cell viability/metabolic activity; it is useful on a large scale mycotoxins screening assay (Dombrink-Kurtzman *et al.*, 1994). Although a variety of mammalian cell cultures were used to detect *Fusarium* mycotoxins in contaminated extracts none of them showed high sensitivity to all of the *Fusarium* mycotoxins tested (Cetin and Bullerman, 2005). The use of human lymphocytes has been described in mycotoxin cytotoxicity test by Maenetje *et al.* (2008). In the past bird, mouse, rat and cattle lymphocytes have already been tested, but they have not been proven to be ideal for routine cytotoxicity testing (Dombrink-Kurtzman *et al.*, 1994; Lioi *et al.*, 2004). Pigs are better test animals, may better correlate with human physiology and are more sensitive to most mycotoxins than other domesticated animals. Our preliminary results obtained in a study on the combined effect of FB_1 and ochratoxin A have shown the feasibility of using isolated pig lymphocytes as test cells in the MTT assay (Mwanza *et al.*, 2009). Mycotoxins have significant economic, scientific and public health significance all over the world. They are biologically active secondary fungal metabolites found as contaminants of food- and feedstuff. *Fusaria* are moulds predominantly producing two types of mycotoxins: the non-oestrogenic trichothecene and the myco-oestrogens including zearalenone and its zearalenol metabolites. These fusa-

1 Kaposvár Univ., Fac. of Animal Science, Guba S. 40, 7400 Kaposvár, Hungary, e-mail: kovacs.melinda@ke.hu
2 Fac. of Health Sciences, Doornfontein Campus, Univ. of Johannesburg, 2028 Doornfontein, Gauteng, South Africa
3 Research Group of Mycotoxins in the Food Chain of the Hungarian Academy of Sciences and Kaposvár Univ., Guba S. 40, 7400 Kaposvár, Hungary

riotoxins are frequently existing contaminants in cereal and other plant products (Scott, 1990). The T-2 and HT-2 toxin belong to a group of trichothecenes (De Nus *et al*., 1996). T-2 toxin is the most acute toxic compound among trichothecenes: it inhibits protein, DNA and RNA synthesis, alters cellular membrane functions, stimulates lipid peroxidation, it is cytotoxic and immunotoxic and induces apoptosis (SCF, 2001). Many *in vitro* studies investigated the cytotoxicity of T-2 and HT-2 (as reported by van der Fels-Klerx and Stratakou, 2010), but the interaction between T-2 and HT-2 has not thoroughly studied yet. There are currently no EC limits for T-2 and HT-2, and there is a lack of a recent overview of the occurrence of these two toxins. In general, a high relationship (ratios between HT-2 and T-2 varied from 1.75 to 7) was found between the levels of the two toxins detected in contaminated commodities (van der Fels-Klerx and Stratakou, 2010).

The aim of the present study was to examine the dose dependent cytotoxic effect of T-2 and HT-2 toxins, single and in combination, using pig lymphocytes.

2 MATERIALS AND METHODS

All chemicals used in this study, including T-2 and HT-2 mycotoxin standards, were obtained from Sigma-Aldrich (Hungary).

The research protocol was reviewed by the Animal Use and Care Administrative Advisory Committee and approved by the Agricultural Administrative Authority (Protocol No.23.1/02322/007/2008). Venous blood from pigs of landrace-large white F_1 genotype (of 4 month age and 24 kg body weight) was taken from the *v. cava cranialis*. The blood was mixed with an equal volume of tissue culture medium consisting of RPMI-1640, then overlaid on Histopaque 1077 and centrifuged as described in Mwanza *et al.* (2009). The interface layer consisting of mononuclear cells (referred to here as lymphocytes) were washed with 9 ml RPMI-1640 at room temperature and centrifuged at 700 g for 15 min, the supernatant was poured, and were washed with 12 ml RPMI-1640 at room temperature and centrifuged at 700 g for 5 min. The pelleted cells were resuspended in 10 ml of complete culture media (CCM), placed in a 96-well culture plates and were cultured at 37 °C in 5% CO_2 humidified incubator for 24h. The CCM was made from 1 ml foetal calf serum (FCS), 100 µl 100U/ml penicillin and 100 µg/ml streptomycin (PenStrep), 150 µl glutamine and made up to a total of 10 ml with RPMI-1640. The paleness of the CCM during the incubation period confirms the growth of cells.

Cell enumeration was carried out by blue dye exclusion method as described by Mwanza *et al.* (2009). Cell count was carried out and % viability was determined as:

% Viability = (viable cell counted (dye excluded) / total no. of cells) * 100

The cell cultures were subdivided and placed in a 96-well culture plates containing 100 µl/well of culture medium. Phytohaemagglutinin (PHA) was used as a cell stimulant (20 µl/well). The mycotoxins were added at various concentrations (0.5, 0.1, 0.05, 0.01 and 0.001 µM of T-2; 1.0, 0.5, 0.2, 0.1 and 0.05 µM of HT-2) and both mycotoxins combined (Table 1), dissolved in ethanol and distilled water. The solvents were also used as controls.

An amount of cell culture medium (RPM-1640) was added to each well to make a total of 400 µl mixture per well. The content was then incubated for 6 and 24 hours and cytotoxicity determined after the incubation time by adding 30 µl MTT solution (Mwanza *et al*., 2009) with mixing. The culture plates were then incubated for 2 h (at 37 °C in 5% CO_2) after which 50 µl dimethylsulphoxide (DMSO) was added to each well and left for further 20 min to solubilise the formazan crystals formed. The optical density (OD) was then read using a micro plate reader at 540 and 620 nm. Cell proliferation as influenced by mycotoxin exposure was determined as percentage viability of the control: % viability = (ODM/ODN) * 100; where ODM is the OD value of the mycotoxin-treated cells; ODN is the OD value of the control (no mycotoxin) cells.

Treatment, concentration and time effect was measured in six replicates.

The cell viability results were analysed by using the Multiway ANOVA procedure of the SPSS 19 software (SPSS Inc., Chicago, IL, USA). The significance of dif-

Table 1: *Toxin treatments used in the MTT assay*

	Concentration categories / toxin concentration (µM)				
Treatments	1	2	3	4	5
T-2	0.5	0.1	0.05	0.01	0.001
HT-2	1.0	0.5	0.2	0.1	0.05
C	0.5 T-2 + 1.0 HT-2	0.1 T-2 + 0.5 HT-2	0.05 T-2 + 0.2 HT-2	0.01 T-2 + 0.1 HT-2	0.001 T-2 + 0.05 HT-2

ferences was tested by Tukey and LSD *post hoc* tests, the level of significance was set at $P < 0.05$. Measured values of combined exposure were compared with the expected values, and the significance of difference between the two values was calculated as described by Šegvić Klarić *et al.* (2012).

3 RESULTS AND DISCUSSION

In the experiment 3 toxin treatments, 5 concentrations of each toxin single and in combination, and 2 exposure times were tested, their effect on cell viability using pig lymphocytes in MTT assay was detemined.

The selection of concentrations was based on data available in the literature concerning cytotoxicity of T-2 and HT-2. In a study of Königs *et al.* (2009) on two human cell lines in primary culture T-2 toxin showed the strongest cytotoxic effect (IC50 values of 0.2 and 0.5 μM) while the metabolites, HT-2 toxin and neosolaniol, revealed weaker cytotoxicity (IC50 values of 0.7–3.0 μM). Similar results were reported by Nielsen *et al.* (2009), according to which IC50 values were 4.4–10.8 nmol/l for T-2, while 7.5–55.8 nmol/l for HT-2 toxin.

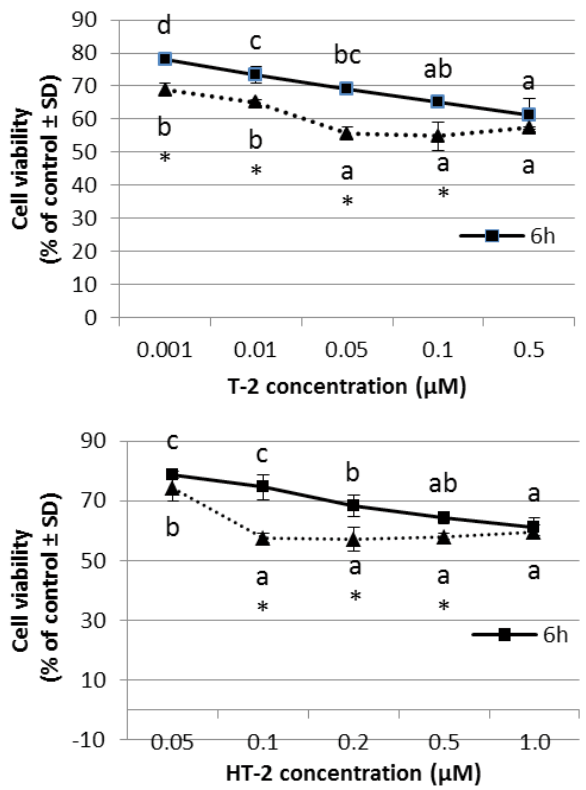

Figure 1: *Cell viability of pig lymphocytes after 6 and 24 h treatment of T-2 (0.001, 0.01, 0.05, 0.1 and 0.5 μM) and HT-2 (0.05, 0.1, 0.2, 0.5 and 1.0 μM) toxin (n = 6; *significant (P < 0.05) difference between values of 6 and 24 h exposure; [a,b,c,d] significant (P < 0.05) difference between concentrations)*

In this study all controls were negative, i.e., their cell viability was taken as 100%.

Both T-2 and HT-2 toxins exerted a dose dependent effect (Fig. 1). After 6 h incubation, the increase in concentration of T-2 from 0.001 to 0.5 μM and HT-2 for 0.05 to 1.0 μM resulted in lower cell viability by 22 and 17%, respectively. After 24 h cell viability was significantly lower compared to values obtained at 6 h, except 0.5 μM T-2 and 0.05, 1.0 μM HT-2, respectively.

After 24 h incubation the increase in T-2 from 0.001 to 0.05 caused 20% decrease in cell viability, thereafter increasing toxin concentration did not result in higher cell death. In case of HT-2 0.1 μM was the highest dose causing significant decrease (by 22%) in cell survival.

Significant ($P < 0.001$) interaction was found between concentration × treatment, and concentration × time, while the time × treatment interaction was not statistically proven ($P = 0.084$).

To estimate the interaction of the two toxins expected values were calculated by Šegvić Klarić *et al.* (2012) and compared to the measured values (Fig. 2). Higher cell viability was measured for all combinations of T-2 and HT-2 except the lowest concentrations applied for 6 h, where the difference between the measured and calculated values was not significant.

When comparing the single and combined effect of the toxins, taking the same concentration categories and incubation time into consideration (Fig. 3), the two toxins caused consistently lower cell survival when applied together than in single administration after 6 h incubation. After 24 h incubation this tendency was not experienced, at concentration 2 the combined application, while at concentration 4 and 5 the single administration of T-2 and HT-2, respectively, resulted in higher cell survival, i.e. was less cytotoxic.

T-2 is rapidly absorbed from the gut, and it is distributed in the organism with little or no accumulation in any specific organs. There is little direct information available on the toxicity of HT-2 toxin alone, according to most data available, T-2 and HT-2 toxin induce toxic effects with similar potency (Joint FAO/WHO …, 2001). Many studies determined a lower cytotoxicity of T-2 metabolites (HT-2, T-2 triol and T-2 tetraol) assuming a detoxification reaction of the organism (Babich and Borenfreund, 1991). As T-2 is rapidly metabolised to HT-2 *in vivo*, the harmful effects of T-2 might partly be attributed to HT-2.

In our study T-2 and HT-2 at the concentrations used, decreased cell viability nearly to the same extend (from 80 to 60%) no significant difference between values of T-2 and HT-2 were obtained within the same con-

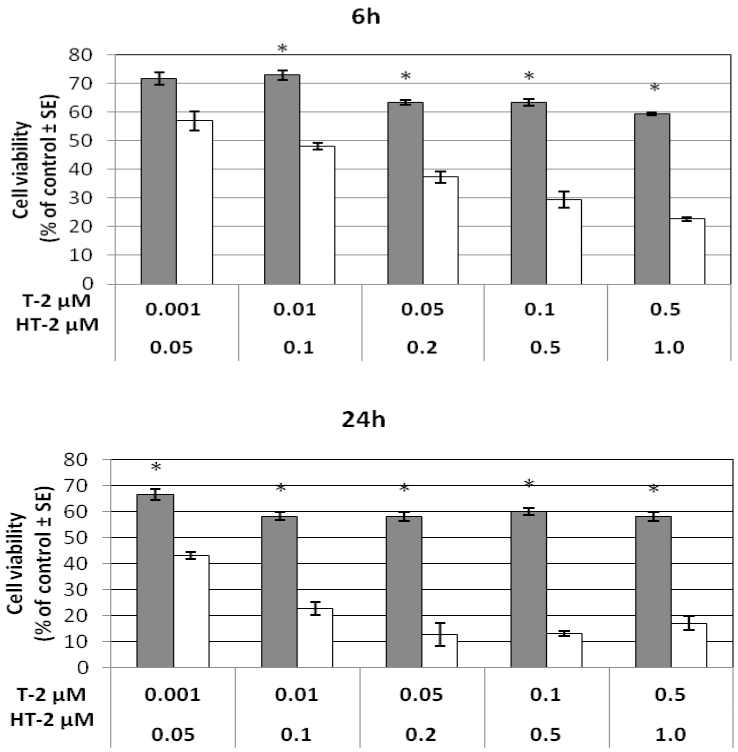

Figure 2: *Cell viability (mean ± SE) of pig lymphocytes after 6 and 24 h of combined treatment with T-2 and HT-2 (grey bars represent the measured values and white bars the expected values. n = 6, *significant (P < 0.05) difference between measured and expected values)*

centration ranges. As HT-2 was added always in higher concentration then T-2, our data supported the lower toxicity of HT-2 compared to T-2 reported previously (Babich and Borenfreund, 1991; Nielsen *et al*., 2009) while are contrary to those of Königs *et al*. (2009) who found similar toxicity of T-2 and HT-2 on human renal tubule epithelial and human lung fibroblast cells.

Further investigations are needed to determine the IC50 for T-2 and HT-2 for pig's lymphocytes, to clarify the type of interaction between the two toxins in lower concentrations, and also to determine if pig lymphocytes do metabolise T-2 to HT-2, as was demonstrated in the case of some cell types (Königs *et al*., 2009).

4 CONCLUSIONS

T-2 and HT-2 toxins had time and dose dependent cytotoxic effect in pigs' lymphocytes measured by MTT assay, HT-2 showing similar cytotoxicity to T-2 but in 2–50-fold higher concentrations. The two toxins caused consistently lower cell survival when applied together than in single administration after 6 h incubation, but for the synergistic effect additional investigations are needed, because the differences in cell viability are not consistent.

5 ACKNOWLEDGEMENT

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6 REFERENCES

Babich H., Borenfreund E. 1991. Cytotoxicity of T-2 toxin and its metabolites determined with the neutral red cell viability assay. Applied and Environmental Microbiology, 57: 2101–2103

Cetin Y., Bullerman L.B. 2005. Cytotoxicity of *Fusarium* mycotoxins to mammalian cell cultures as determined by the MTT bioassay. Food and Chemical Toxicology, 43: 755–764

De Nus M., Rombouts F., Notermans S. 1996. *Fusarium* molds and their mycotoxins. Journal of Food Safety, 16: 15–58

Dombrink-Kurtzman M.A., Bennett G.A., Richard J.L. 1994. An optimized MTT bioassay for determination of cytotoxicity of fumonisins in turkey lymphocytes. Journal of AOAC International, 77, 2: 512–516

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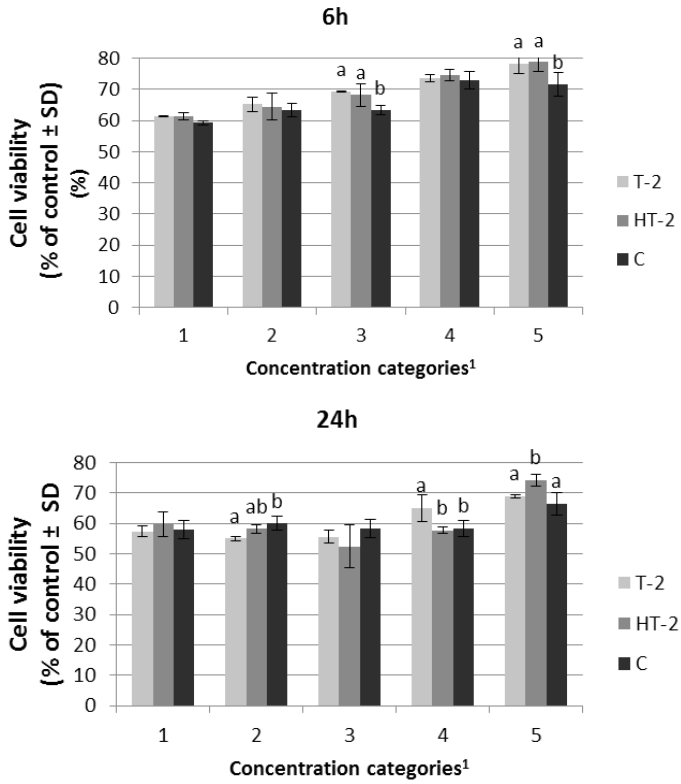

Figure 3: *Comparison of single and combined effect of T-2 and HT-2 toxins ([1] five decreasing toxin concentrations were applied both single and in combination (see Materials and methods); C = combined application of T-2 and HT-2; [a,b] significant difference between treatments (T-2, HT-2 and C) with the same concentration category (from 1 to 5) and incubation time (P < 0.05)*

FA) 2001. Safety evaluation of certain mycotoxins in food. WHO Food Additives Series, No. 47/FAO Food and Nutrition Paper 74. http://www.inchem.org/documents/jecfa/jecmono/v74je01.htm

Königs M., Mulac D., Schwerdt G., Gekleb M., Humpf H.U. 2009. Metabolism and cytotoxic effects of T-2 toxin and its metabolites on human cells in primary culture. Toxicology, 258: 106–115

Lioi M.B., Santoro A., Barbieri R., Salzano S., Ursini M.V. 2004. Ochratoxin A and zearalenone: a comparative study on genotoxic effects and cell death induced in bovine lymphocytes. Mutation Research, 557: 19–27

Maenetje P.W., de Villiers N., Dutton M.F. 2008. The use of isolated human lymphocytes in mycotoxin cytotoxicity testing. International Journal of Molecular Sciences, 9: 1515–1526

Mwanza M., Kametler L., Bonai A., Rajli V., Kovacs M., Dutton M.F. 2009. The cytotoxic effects of fumonisin B_1 and ochratoxin A on human and pig lymphocytes using methyl thiazol tetrazolium (MTT) assay. Mycotoxin Research, 25, 4: 233–238

Nielsen C., Casteel M., Dider A., Dietrich R., Märtlbauer E. 2009. Trichothecene-induced cytotoxicity on human cell lines. Mycotoxin Research, 25: 77–84

Šegvić Klarić, M., Zeljezic, D., Rumora, L., Peraica, M., Pepeljnjek, S., Domijan, A.M. 2012. A potential role of calcium in apoptosis and aberrant chromatin forms in porcine kidney PK15 cells induced by individual and combined ochratoxin A and citrinin. Archives of Toxicology, 86, 1: 97–107

SCF – Scientific Committee on Food 2001. Opinion of the Scientific Committee on Food on Fusarium toxins. Part 5: T-2 toxin and HT-2 toxin. European Commission, Brussels, Belgium. http://ec.europa.eu/food/fs/sc/scf/out88_en.pdf

Scott P.M. 1990. Trichothecenes in grains. Cereal Foods World, 35: 661–666

Van der Fels-Klerx H.J., Stratakou I. 2010. T-2 toxin and HT-toxin in grain and grain-based commodities in Europe: occurrence, factors affecting occurrence, co-occurrence and toxicological effects. World Mycotoxin Journal, 3, 4: 349–367